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Bearded Family Members Inhibit Neuralized-Mediated Endocytosis and Signaling Activity of Delta in *Drosophila*

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Summary

Endocytosis of Notch receptor ligands in signaling cells is essential for Notch receptor activation. In *Drosophila*, the E3 ubiquitin ligase Neuralized (Neur) promotes the endocytosis and signaling activity of the ligand Delta (Dl). In this study, we identify proteins of the Bearded (Brd) family as interactors of Neur. We show that Tom, a prototypic Brd family member, inhibits Neur-dependent Notch signaling. Overexpression of Tom inhibits the endocytosis of Dl and interferes with the interaction of Dl with Neur. Deletion of the *Brd* gene complex results in ectopic endocytosis of Dl in dorsal cells of stage 5 embryos. This defect in Dl trafficking is associated with ectopic expression of the *single-minded* gene, a direct Notch target gene that specifies the mesectoderm. We propose that inhibition of Neur by Brd proteins is important for precise spatial regulation of Dl signaling.

Introduction

Cell surface receptors of the Notch/Lin-12 family mediate short-range signaling that underlies many developmental decisions in Metazoa (Lai, 2004). Notch receptors are activated by transmembrane ligands of the DSL (*D*elta, *S*errate, *L*AG-2) family. Interaction of DSL ligands with Notch promotes the proteolytic cleavage of Notch and the release of the *N*otch *i*ntrac*e*llular *d*omain (NICD) that acts in the nucleus as a transcriptional coactivator.

The role of Notch in regulating cell fate decisions has been particularly well studied in the context of sensory organ formation in *Drosophila*. Detailed studies have shown that Notch mediates lateral inhibition, a patterning process that regulates the singling out of regularly spaced sensory organ precursor cells (SOP) within groups of equipotent cells known as the proneural clusters. In the absence of Notch signaling, all proneural cluster cells adopt a neural fate, indicating that Notch activation is required to limit the number of proneural cluster cells becoming SOPs. Once singled out, SOPs divide asymmetrically to generate two secondary precursor cells, pIIa and pIIb. In the absence of Notch, both SOP daughter cells become pIIb, indicating that Notch activation is required for the specification of the pIIa fate.

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Notch receptor activation is regulated in space and time by several distinct mechanisms (Schweisguth, 2004). Many recent studies have highlighted the essential yet not fully understood role that endocytosis plays in DSL signaling (Le Borgne et al., 2005a). At least three E3 ubiquitin ligases regulate the endocytosis of DSL ligands in vertebrate and invertebrate species: Neuralized (Neur), Mind bomb 1 (Mib1 in *Drosophila* [also previously named D-mib]; Mind bomb in vertebrates), and Mind bomb 2 (Mib2 in *Drosophila*; Skeletrophin or Mind bomb-like in vertebrates) (Deblandre et al., 2001; Itoh et al., 2003; Koo et al., 2005a, 2005b; Lai et al., 2001, 2005; Le Borgne et al., 2005b; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005; Takeuchi et al., 2005; Wang and Struhl, 2005; Yeh et al., 2001). Neur contains two Neur homology repeats (NHR) of unknown function and one C-terminal catalytic RING domain. Mib1 and Mib2 are related multidomain proteins that, like Neur, contain a C-terminal catalytic RING domain but have otherwise different domain composition compared to Neur. Despite these structural differences, Neur and Mib1 have similar molecular activities. Neur and Mib1 are able to bind Dl and Ser and promote their ubiquitination and endocytosis. It is thought that Neur and Mib1 (and possibly Mib2) monoubiquitinate Dl and Ser and that monoubiquitination acts as a signal for endocytosis and epsin-mediated sorting (Lai et al., 2001; Le Borgne and Schweisguth, 2003; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005; Wang and Struhl, 2004, 2005).

Despite these similar modes of action, Neur and Mib1 have distinct functions in vivo, likely due to differences in expression pattern (Lai et al., 2005; Lai and Rubin, 2001; Le Borgne et al., 2005b; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). In particular, the activity of the *neur* gene is essential for Dl signaling during lateral inhibition and binary fate decisions in sensory lineages, whereas the activity of the *mib1* gene plays only a minor role in these two processes. Conversely, *mib1* activity is required for Ser and, to a possibly lesser extent, Dl signaling for the specification of the wing margin, whereas *neur* activity is dispensable for this process. Nevertheless, the lack of domain similarity between Neur and Mib1 raises the possibility that, in addition to regulation at the transcriptional level, specific mechanisms regulating the activity of either Neur or Mib1 may exist.

To identify potential regulators of Neur, a yeast two-hybrid screen was conducted using Neur as bait. Several members of the Bearded (Brd) family were identified as interactors of Neur. Proteins of the Brd family are small (70–285 amino acids) proteins of unknown activity. The founding member of this gene family, *Brd*, was discovered through a gain-of-function mutation, *Brd[1]*, that causes an increase in bristle density suggestive of a failure of Notch signaling (Leviten and Posakony, 1996). An additional nine genes encoding proteins similar to *Brd* have been found in the *Drosophila* genome: *Ocho*, *Tom*, *BobA*, *-B*, *-C* (the *Bob* genes are identical at the amino acid level; we will therefore refer to them

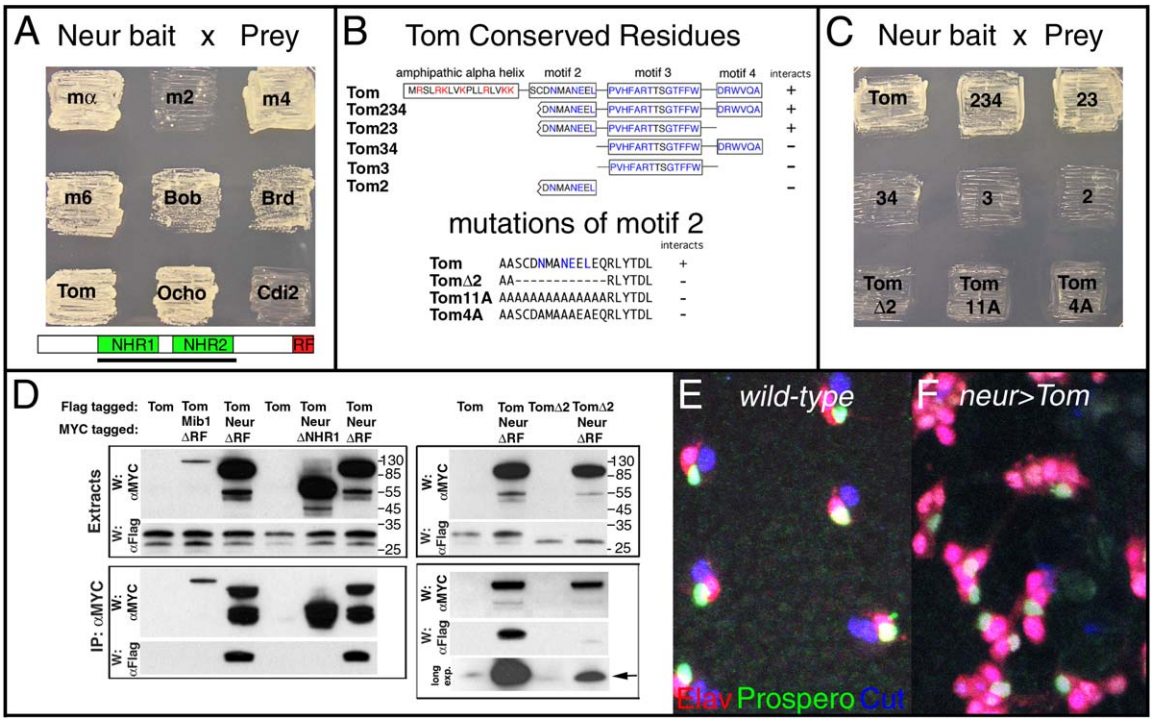

Figure 1. Proteins of the Brd Family Interact with Neur

(A) The *Brd* genes *mα*, *m4*, *m6*, *Bob*, *Brd*, *Ocho*, and *Tom*, but not *m2*, interact with Neur based on *LEU2* activation in the yeast two-hybrid assay. Cdi2 is a negative control.

(B) Structure of the full-length, truncated, deleted, and mutated versions of Tom assayed in (C).

(C) Interactions of the various versions of Tom (see [B]) with Neur using the two-hybrid assay.

(D) MYC-tagged NeurΔRF, NeurΔNHR1, and Mib1ΔRF were immunoprecipitated with rabbit anti-MYC. Flag-tagged Tom coimmunoprecipitated with MYC-tagged NeurΔRF but not with MYC-tagged NeurΔNHR1 or Mib1ΔRF (left panel). Coimmunoprecipitation of Flag-tagged Tom with MYC-tagged NeurΔRF was greatly diminished (but not abolished) upon deletion of motif 2 in TomΔ2 (right panel). Two different exposures of the same blot are presented to show the weak binding of TomΔ2 (arrow) to NeurΔRF.

(E and F) Sensory organs in nota of either wild-type (E) or *neur>Tom* (F) pupae at 24 hr after puparium formation. Each wild-type sensory organ is composed of four Cut-positive (blue) cells and includes one Prospero-positive (green) sheath cell and one Elav-positive (red) neuron. Overexpression of Tom results in the determination of additional SOPs (not shown) and in the partial transformation of sensory organs cells into neurons.

collectively as "*Bob*"), *mα*, *m2*, *m4*, and *m6* (Lai et al., 2000a, 2000b; Leviten et al., 1997; Wurmbach et al., 1999; Zaffran and Frasch, 2000). The *Brd*, *Ocho*, *Tom*, and *Bob* genes are clustered in a complex, the *Brd* complex (*Brd-C*), whereas the *mα*, *m2*, *m4*, and *m6* genes are found within the *Enhancer of split* complex (*E[spl]-C*). Consistent with a function in sensory organ development, the *Brd*, *mα*, *m4*, and *Ocho* genes are expressed within proneural clusters under the control of proneural factors and Notch signaling. Additionally, overexpression studies indicate that the *Brd* family genes, with the exception of *m2*, may act to inhibit Notch signaling (Castro et al., 2005; Lai et al., 2000a, 2000b; Leviten et al., 1997; Singson et al., 1994; Wurmbach et al., 1999; Zaffran and Frasch, 2000). However, loss of *Brd* or *Tom* function did not result in phenotypes associated with altered Notch signaling, suggesting functional redundancy within this family (Leviten et al., 1997; Zaffran and Frasch, 2000). Thus, the mechanism whereby these genes antagonize Notch and the functional significance of this inhibition is not known.

We show here that proteins of the Brd family inhibit the Neur-mediated endocytosis of Dl and that inhibition of Neur by Brd proteins is important for precise allocation of cell fates along the dorsal-ventral (DV) axis in the early embryo. Additionally, we propose that Brd proteins may also inhibit Neur-mediated endocytosis of Dl during lateral inhibition.

Results

Bearded Family Members Interact with Neuralized

In order to identify regulators of Neur, a yeast two-hybrid screen was conducted using as bait the conserved central domain of Neur that comprises the two NHRs (Figure 1A). Eighty-four cDNAs were identified, of which 62 encoded members of the *Brd* gene family: *Ocho* (39 times [×]), *Tom* (12×), *m4* (7×), *Brd* (2×), and *Bob* (2×). Interaction of Neur with m6, mα, and m2 was tested directly. The m6 and mα proteins, but not m2, interacted with Neur in this assay (Figure 1A). Tom also interacted with full-length Neur (data not shown; Giot et al., 2003). We conclude that all Brd family members, with the exception of m2, interact with Neur.

Most Brd proteins share four conserved motifs (Figure 1B): a lysine-rich N-terminal region predicted to form an amphipathic α helix; a short NxxNExLE motif (see Figure S1 in the Supplemental Data available with

this article online; this motif is hereafter referred to as motif 2) found in all proteins except m2; and two C-terminal motifs (motifs 3 and 4) found in only a subset of the Brd family members (Lai et al., 2000b). Motifs 2 and 3 are the most-conserved motifs among insect Brd homologs (Figure S1). Because Brd and Bob lack motifs 3 and 4, these motifs cannot be strictly required for interaction with Neur. Additionally, clones encoding N-terminally truncated Tom proteins (amino acids 75–158, 58–158, 54–158, and 29–158 of Tom) were recovered in our screen, indicating that the N-terminal region predicted to form an amphipathic helix (amino acids 26–43 of Tom) is not necessary for interaction with Neur. Thus, motif 2 is the only conserved motif present in all the clones that interact with Neur. It is also absent from m2, which is the only family member that does not interact with Neur and fails to inhibit Notch when overexpressed. Deletion and point mutation analysis of Tom further demonstrates that motif 2 is important for Neur binding. First, truncated versions of Tom lacking motif 2 did not interact with Neur in the two-hybrid assay. Second, internal deletion of this motif strongly impaired interaction in the two-hybrid assay. Third, alanine substitution of either 11 or 4 residues of motif 2 also impaired interaction (Figure 1C). We conclude that motif 2 is important for Neur binding.

To verify the interaction of Neur and Tom, we conducted coimmunoprecipitation experiments in HEK293 cells. A MYC-tagged version of Neur deleted of its RING finger (NeurΔRF) was used in this assay, as previous studies had suggested that this form of Neur is more stable (Lai et al., 2001). NeurΔRF was found to immunoprecipitate Flag-tagged Tom protein (Figure 1D). This confirms that Neur physically interacts with Tom. A version of Tom that carries a deletion of motif 2 (TomΔ2) was found to interact only weakly with Neur (Figure 1D). While this result indicates that motif 2 contributes to the binding of Tom to Neur, it also suggests that sequence motifs other than motif 2 may be involved in the interaction of Tom with Neur. Consistent with this idea, motif 2 was insufficient for interaction with Neur in the two-hybrid assay (Figure 1C). Deletion of the NHR1 domain abolished the interaction of Neur with Tom, suggesting that this domain of Neur is necessary for interaction (Figure 1D). Consistent with this, the first NHR of chick cNeur1 was sufficient for interaction with *Drosophila* Tom (data not shown). Finally, MYC-tagged Mib1 deleted of its C-terminal RING finger (Mib1ΔRF) did not interact with Tom. While we note that Mib1ΔRF is expressed at much lower levels than NeurΔRF, Tom is not detected in the Mib1 immunoprecipitate upon longer exposure of the blot (data not shown). This result indicates that Tom specifically interacts with Neur.

Together, these results show that Brd family members bind Neur. We will hereafter refer to the *Brd* family of genes to mean *Brd*, *BobA*, *-B*, *-C*, *Ocho*, *Tom*, *mα*, *m4*, and *m6*; we exclude *m2* from this group as no interaction with Neur was detected.

Overexpression of Tom Specifically Blocks Neur-Dependent Notch Signaling

Recent studies have shown that the activity of *neur* is required to regulate only a subset of Notch signaling events, with *mib1* acting in a complementary manner to regulate a distinct subset of Notch signaling events (Lai et al., 2005; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). We therefore examined whether the overexpression of genes of the *Brd* family interferes with all Notch signaling events or only with Neur-dependent ones. Consistent with previously published results, we found that the forced expression of different *Brd* family members during sensory organ development resulted in a neurogenic phenotype (Lai et al., 2000a, 2000b; Leviten et al., 1997; Wurmbach et al., 1999; Zaffran and Frasch, 2000). Because the strongest gain-of-function phenotype was seen upon the overexpression of *Tom*, we used *Tom* in all of our experiments. The overexpression of *Tom* in sensory organ cells using the neur^{P72}Gal4 driver resulted in the specification of extra SOPs and extra neurons at the expense of the other cell fates in the lineage (Figures 1E and 1F). In contrast, the overexpression of *Tom* in wing imaginal discs (using *dpp*-Gal4, *ptc*-Gal4, or *Ser*-Gal4) had no effect on the specification of wing margin cells or on wing pouch growth (see below; data not shown). Thus, Tom can inhibit Neur-dependent signaling but has no detectable effect on Mib1-dependent signaling. These results are consistent with Brd family members acting as specific antagonists of Neur.

Overexpression of Tom Inhibits Dl Endocytosis

If Brd family members antagonize Notch signaling by inhibiting Neur, then overexpression of *Brd* genes should block Dl endocytosis. To test this prediction, Tom was expressed in sensory organ cells using neur^{P72}Gal4 and the distribution of Dl was examined on fixed nota. The overexpression of Tom led to an accumulation of Dl at the plasma membrane of SOP progeny cells (Figures 2A and 2B′). This accumulation of Dl did not result from an increase in *Dl* expression as no upregulation of *Dl-lacZ* expression was observed upon Tom overexpression using the *ap*-Gal4 driver (Figures 2F and 2G′; see Figure 2E for *ap*-Gal4 expression pattern).

To test whether the plasma membrane accumulation of Dl is due to a block in Dl endocytosis, we used an anti-Dl antibody uptake assay (Le Borgne and Schweisguth, 2003). Briefly, dissected nota were incubated for 15 min with anti-Dl antibodies that recognize the extracellular portion of Dl, washed briefly, and then fixed. Internalized anti-Dl antibodies were then detected using secondary antibodies. As previously reported (Le Borgne and Schweisguth, 2003), anti-Dl antibodies were efficiently endocytosed by wild-type sensory organ cells, whereas loss of *neur* activity resulted in an accumulation of anti-Dl antibodies at the cell surface (Figures 2C and 2C′). Overexpression of Tom (using *ap*-Gal4) resulted in similar defects in anti-Dl antibody localization: anti-Dl antibodies remained largely bound to the surface of sensory cells (Figures 2D and 2D′). We conclude that overexpression of Tom inhibits the endocytosis of Dl. Thus, Brd family members block Neur-dependent Notch signaling by inhibiting the endocytosis of Dl.

Tom Does Not Downregulate Neur Protein Levels

Inhibition of Neur by the *Brd* genes could result from downregulation of Neur protein levels. To address this, we compared the levels of endogenous Neur protein in

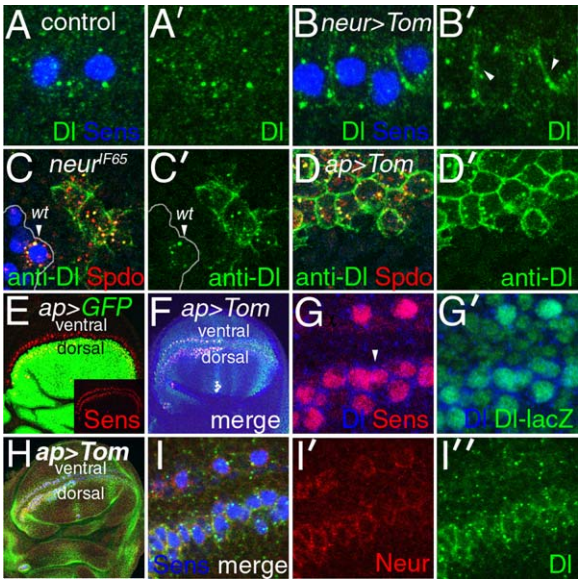

Figure 2. Overexpression of Tom Inhibits the Endocytosis of Dl

(A and A′) Dl (green) accumulates into endocytic vesicles in pIIa and pIIb cells (marked by Senseless [Sens] in blue) in control neur^{p72}Gal4 pupae.

(B and B′) Overexpression of Tom using neur^{p72}Gal4 results in the accumulation of Dl at the plasma membrane of SOP daughter cells (arrowheads in [B′]; two pairs of secondary precursor cells are shown).

(C–D′) Endocytosis of Dl in sensory cells (marked by Sanpodo [Spdo] in red) was monitored using an antibody uptake assay (anti-Dl antibodies in green).

(C and C′) GFP-positive wild-type sensory cells (arrowhead) efficiently endocytosed Dl. In contrast, anti-Dl antibodies accumulated at the surface of the GFP-negative *neur^{IF65}* mutant sensory cells (GFP [red] was used as a clonal marker; clonal boundary is outlined).

(D and D′) Overexpression of Tom in *ap>Tom* pupae blocked the endocytosis of Dl in pupal nota, as revealed by the accumulation of anti-Dl antibodies at the surface of sensory cells.

(E) The domain of *ap*-Gal4 expression is revealed in *ap>GFP* wing imaginal discs. GFP (green) expression is restricted to the dorsal compartment. SOPs on either side of the wing margin are marked by Sens (red).

(F–G′) Expression of Tom in *ap>Tom* wing imaginal discs led to the specification of too many SOPs in the dorsal compartment (arrowhead in [G]). It did not, however, detectably change the level of *Dl-lacZ* expression (green in [F] and [G′]) in SOPs (Dl in blue).

(H–I″) Overexpression of Tom in the dorsal compartment of *ap>Tom* wing discs did not detectably reduce the levels of Neur protein (red in [I′]). Dl (green in [I″]) accumulated at the cell surface of dorsal SOPs.

SOPs located on either side of the wing margin in discs overexpressing Tom only in dorsal cells using the *ap*-Gal4 driver. Neur protein levels appear very similar in Tom-overexpressing dorsal and control ventral SOPs (Figures 2H and 2I″). Thus, Brd proteins do not appear to downregulate Neur activity by affecting protein accumulation.

Tom Inhibits a Dominant-Negative Version of Neur

A second possibility is that Brd proteins inhibit the catalytic E3 ligase activity of Neur. If this were the case, then inhibition of Neur by Brd should require Neur to be catalytically active. To test this possibility, a cysteine-to-serine mutation, previously shown to abolish in vitro E3 ubiquitin ligase activity (Yeh et al., 2001), was introduced at position 701 of the RING domain to generate NeurC701S. NeurC701S failed to suppress the embryonic *neur* mutant phenotype (S. Hamel and F.S., unpublished results). Expression of NeurC701S in the wing using *Ser*-Gal4 inhibited the formation of the wing margin (Figure 3A). A similar effect has previously been reported for NeurΔRF (Lai and Rubin, 2001). Because wing margin formation is Neur independent but Mib1 dependent, we suggest that NeurC701S may act through binding or sequestering a substrate or cofactor of Neur that is also important for Mib1 activity. This phenotype resulting from the overexpression of NeurC701S allowed us to test whether the catalytic activity of Neur is required for inhibition by the *Brd* genes. Expression of Tom strongly suppressed the wing margin phenotype associated with NeurC701S (Figure 3A). Tom similarly suppressed the NeurΔRF overexpression phenotype, indicating that the RING domain is not required (data not shown). In contrast, overexpression of Tom had no effect on the inhibition of wing margin development by Mib1C1205S, an inactive version of Mib1 that carries a cysteine-to-serine mutation in its C-terminal RING finger (Figure 3A). This is consistent with our results showing that Tom specifically binds and inhibits Neur but not Mib1. We conclude that Brd proteins can antagonize Neur in a manner that does not require its catalytic E3 ligase activity.

Tom Inhibits the Binding of Dl with Neur

Next, we tested the hypothesis that Tom inhibits the binding of Neur to its substrate. Consistent with this possibility, previous studies have indicated that interaction of Dl with Neur requires the NHR1 domain of Neur (Lai et al., 2001) that was shown here to be important for the binding of Tom. The effect of Tom on the interaction of Dl with NeurΔRF was examined using coimmunoprecipitation. The amount of Dl immunoprecipitated with NeurΔRF was reduced in the presence of Tom (Figure 3B). Tom had no detectable effect on the amount of Neur or Dl present in the extracts. These results suggest that Tom can inhibit the interaction of Dl with Neur. We propose that Brd proteins antagonize Neur by inhibiting its ability to interact with its substrate Dl.

Deletion of the *Brd-C* Results in Defects in Dl Localization in the Early Embryo

We then investigated the biological significance of the inhibition of Neur by Brd family members in the embryo using deficiencies. The *E(spl)-C* encodes seven bHLH transcription factors required for Notch signaling (Knust et al., 1992), thereby preventing us from analyzing the specific roles of the *mα*, *m4*, and *m6* genes in Notch signaling. We therefore restricted our analysis to the *Brd-C*.

During early embryogenesis, Neur-dependent Notch signaling plays an important role in DV patterning as it regulates the expression of the Notch target gene *single-minded* (*sim*) in a single row of mesectodermal cells abutting the mesoderm (Cowden and Levine, 2002; Martin-Bermudo et al., 1995; Morel et al., 2003; Morel and Schweisguth, 2000). As described previously, the *neur* gene is strongly expressed in the mesoderm of stage 5 embryos. It is, however, not restricted to this tissue, and *neur* transcripts are also detected in the

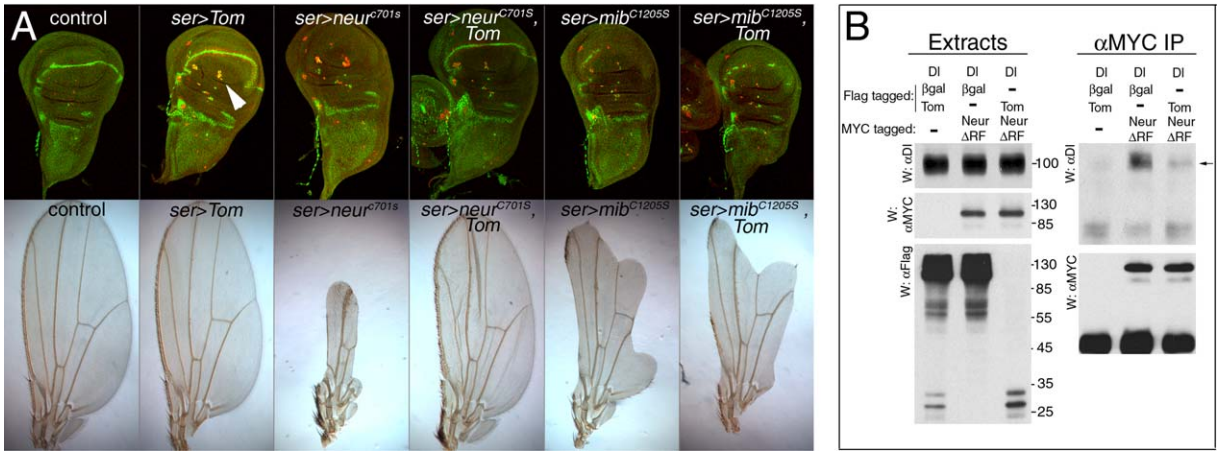

Figure 3. Mode of Action of Brd Proteins

(A) Genetic interactions between Tom and the catalytically dead versions of Neur (NeurC701S) and Mib1 (Mib1C1205S) were studied by overexpression using *Ser*-Gal4. Phenotypes were examined in both wing imaginal discs (top panels) and adult wings (bottom panels). Imaginal discs were stained with Cut (green) and Sens (red) to mark the wing margin and the sensory cells, respectively. Overexpression of Tom had no effect on Cut expression at the wing margin or on growth of the wing pouch, both of which depend on *mib1* activity. However, it blocked lateral inhibition, a *neur*-dependent process (arrowhead). Overexpression of NeurC701S and Mib1C1205S strongly inhibited wing margin specification as well as wing growth. Overexpression of Tom suppressed the dominant-negative effect of NeurC701S but not that of Mib1C1205S.

(B) Tom inhibits the binding of Neur to Dl. HEK293 cells were transfected with plasmids encoding Polyoma-tagged Dl, MYC-tagged NeurΔRF, Flag-tagged Tom, and Flag-tagged β-Gal as indicated above each lane. Western blot analysis of the extracts is shown in the left panels (1/30 of the total extract was loaded in each lane; molecular weight markers are indicated). Western blot analysis of the anti-MYC immunoprecipitated materials is shown in the right panels. Dl coimmunoprecipitated with Neur, as previously described by Lai et al. (2001). Expression of Tom reduced the amount of Dl bound to Neur (arrow, right panel).

mesectoderm as well as in more dorsal tissues (Boulianne et al., 1991) (Figure S2). In contrast, the *Tom*, *Brd*, and *Bob* genes are strongly expressed in lateral and dorsal regions and are not detectably expressed in the mesoderm (Nagel et al., 2000; Zaffran and Frasch, 2000) (Figure S2). *Ocho* transcripts are detected in a single row of cells that presumably correspond to the mesectoderm. Thus, all genes of the *Brd-C* are expressed in cells that have low levels of *neur* transcripts, whereas they are not detectably expressed in mesodermal cells that have high levels of *neur* transcripts in stage 5 embryos. Previous studies have also shown that Dl accumulates in dots in the mesoderm of stage 5 embryos and that zygotic *neur* activity is required for this accumulation (Kooh et al., 1993; Morel et al., 2003) (Figures 4A and 4B), suggesting a model whereby Neur regulates the endocytosis of Dl in ventral cells and Dl signals from the mesoderm to activate Notch in adjacent cells (Morel et al., 2003). This model, however, does not explain the tight restriction of Dl endocytosis and signaling to the mesoderm.

The possible role of the *Brd-C* on Dl endocytosis was studied at midcellularization, that is, at the onset of *sim* transcriptional activation and at a stage when Dl accumulates almost exclusively at the plasma membrane in nonmesodermal cells, whereas in mesodermal cells, Dl is mostly found in intracellular dots where it colocalizes with Notch extracellular epitopes (NECD) (Figure 4A). Deletion of the *Brd-C* results in the accumulation of Dl and NECD in basal dots all around the embryo, including in the dorsal-most cells in *Df(3L)Brd12*, *Df(3L)ED217*, and *Df(3L)Brd15* embryos (Figure 4C; data not shown). Because these three deficiencies are large, it is conceivable that genes located outside the *Brd-C* contribute to this phenotype. To rule out this possibility, we have used Flp-mediated recombination (Thibault et al., 2004) to generate a small 38 kb deletion that removes the entire *Brd-C* and also truncates CG13466, a predicted gene of unknown function (Figure S3). Embryos homozygous for this deletion, called *Df(3L)Brd-C1*, exhibited similar defects in Dl localization (Figures S4C and S4D). CG13466 is not detectably expressed in the early embryo prior to stage 11 (http://www.fruitfly.org/cgi-bin/ex/insitu.pl), and is therefore unlikely to play a role in Dl endocytosis during stage 5. We therefore conclude that the activity of the *Brd-C* is required to restrict the accumulation of Dl in basal dots to the presumptive mesoderm. Consistent with a role of the *Brd-C* in inhibiting the accumulation of Dl in basal dots, the overexpression of Tom in the early embryo using a maternal Gal4 driver led to a persistent membrane accumulation of Dl in ventral cells that correlates with a decrease in the accumulation of Dl in basal dots (Figure S4). Thus, the ectopic expression of Tom appears to block the endocytosis of Dl. This effect is similar to the one observed in sensory organ cells (Figure 2).

Deletion of the *Brd-C* Results in Ectopic Dl Endocytosis

To test whether Dl accumulates into basal dots as a result of its endocytosis from the plasma membrane, we adapted for the embryo the anti-Dl antibody uptake assay used above in the notum (Le Borgne and Schweisguth, 2003). Briefly, anti-Dl antibodies were injected into the perivitelline space and embryos were fixed for 10–15 min following injection. Internalized antibodies were then detected using secondary antibodies. Anti-Dl antibodies were efficiently internalized by ventral cells (Figures 5A and 5A′; n = 21/21; all 21 injected embryos showed many ventral dots) but were poorly taken up

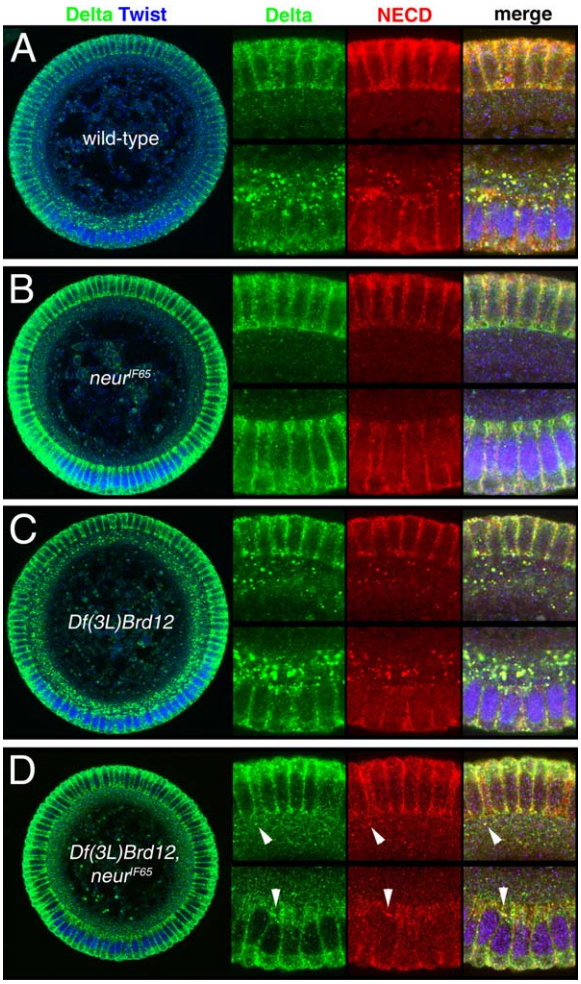

Figure 4. The Subcellular Distribution of Dl Depends on the Activity of the *Brd-C*

Cross-sectional views of stage 5 (midcellularization) embryos stained for Dl (green), NECD (red), and Twist (blue). Higher magnification views showing the dorsal- (upper panels) and ventral-most (lower panels) regions of the same embryos are shown on the right.
(A) Dl colocalizes with NECD in punctate dots restricted to the mesoderm in wild-type embryos.
(B) *neur*^{IF65} mutant embryos are nearly completely devoid of Dl- and NECD-positive dots.
(C) *Df(3L)Brd12* deficiency embryos exhibit Dl- and NECD-positive dots all around the embryo. Note, however, that there are still more dots in ventral cells than in dorsal cells.
(D) *Df(3L)Brd12, neur*^{IF65} double mutant embryos lack most Dl- and NECD-positive dots. Note the weak Dl-NECD punctate staining (arrowheads) seen in *Df(3L)Brd12, neur*^{IF65} double mutants that is not detected in zygotic *neur*^{IF65} single mutants. We suggest that a low level of Dl endocytosis independent of the zygotic activity of the *neur* gene can be revealed in *Df(3L)Brd12* embryos, and that this endocytosis may be dependent on maternally provided Neur.

by dorsal cells (Figures 5C–5C″; n = 1/11; a single embryo out of 11 injected embryos showed a few dots and was scored positive). Internalized anti-Dl antibodies colocalized with Dl in basal vesicles in ventral mesodermal cells (Figure S5). Internalization by ventral cells of anti-Dl antibodies depended on zygotic *neur* activity (Figures 5B and 5B′; n = 0/5). These results indicate that the endocytosis of Dl is both *neur* dependent and mesoderm specific at stage 5.

We then tested whether the activity of the *Brd-C* is required to inhibit the endocytosis of Dl in dorsal cells. Anti-Dl antibodies were efficiently endocytosed by dorsal cells in *Df(3L)Brd12* embryos (Figures 5D–5D″; n = 4/4). This contrasts with the inefficient uptake of anti-Dl antibodies by dorsal cells in wild-type embryos (Figures 5C–5C″; n = 1/11). We conclude that the accumulation of Dl in basal dots seen in *Df(3L)Brd12* embryos results from an ectopic endocytosis of Dl and that Brd family members prevent Dl endocytosis in dorsal and lateral cells.

Ectopic Dl Endocytosis in *Brd-C* Embryos Is *neur* Dependent

The ectopic endocytosis of Dl seen in *Df(3L)Brd12* embryos suggests that genes of the *Brd-C* inhibit the activity of Neur in nonmesodermal cells. If this interpretation is correct, then the ectopic vesicular accumulation of Dl seen in *Brd-C* mutant embryos should depend on *neur* activity. To test this prediction, we examined the localization of Dl in *neur*^{IF65}*, Df(3L)Brd12* double mutant embryos (Figure 4D). At midcellularization, the number and intensity of the Dl-NECD dots were greatly reduced in dorsal cells of double mutant embryos relative to *Df(3L)Brd12* embryos. This indicates that the ectopic endocytosis of Dl seen in *Df(3L)Brd12* embryos is primarily dependent on the zygotic activity of the *neur* gene.

Deletion of the *Brd-C* Results in Ectopic Notch Target Gene Activation

The *neur*-dependent endocytosis of Dl in the mesoderm is thought to be responsible for Notch activation and *sim* expression in the mesectoderm (Figure 6A). The *sim* gene, a direct Notch target, specifies the mesectoderm (Morel and Schweisguth, 2000; Nambu et al., 1990). Following mesoderm invagination, the mesectoderm forms the ventral midline and gives rise to specific midline neuroblasts. Loss of *neur* activity leads to a loss of *sim* expression in stage 5 embryos (Figure 6B; see also Martin-Bermudo et al., 1995). In contrast, loss of *Brd-C* activity leads to the ectopic expression of *sim* in a few cells dorsal to the mesectoderm at stage 5 (Figures 6C and 6D), which correlates with the specification of extra *sim*-positive midline cells in stage 8 embryos (Figures 6F–6H). A qualitatively similar effect was previously reported for the uniform expression of an activated version of Notch (Morel and Schweisguth, 2000). We conclude that loss of *Brd-C* activity leads to ectopic Notch activity. Interestingly, a partial suppression of the *neur*^{IF65} mutant phenotype was observed upon removal of the *Brd-C* (Figure 6E). We interpret this result to suggest that a low level of Notch activation independent of the zygotic activity of the *neur* gene can be observed in *Df(3L)Brd12* embryos. This activation of Notch may be dependent on maternally provided Neur.

We conclude that the activity of the *Brd-C* is required to restrict Notch activation along the DV axis and propose that ectopic Dl endocytosis results in ectopic Notch activation.

Discussion

Activation of DSL signaling by Neur is regulated by multiple mechanisms. A first level of regulation operates at the transcriptional level, both along the DV axis in the

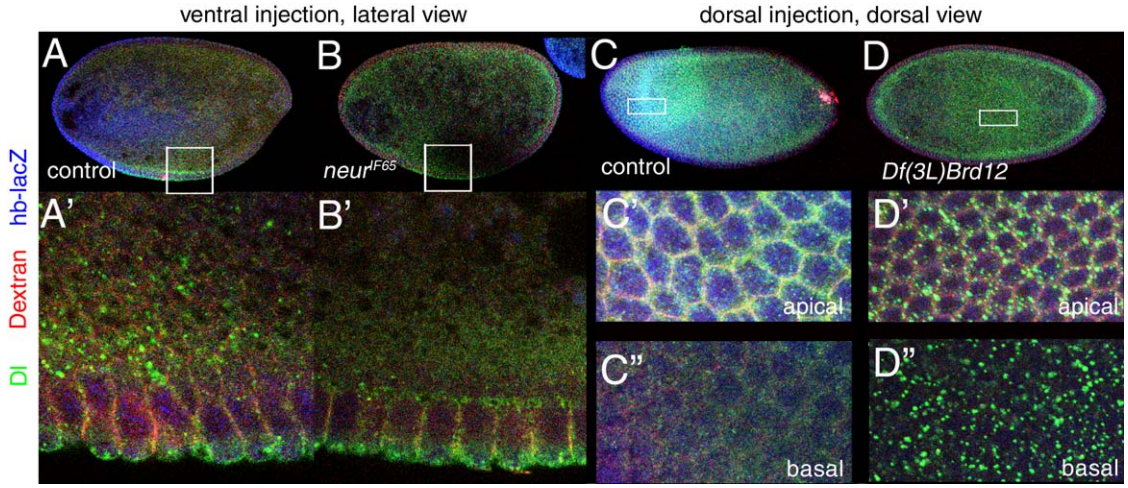

Figure 5. The *Brd-C* Is Required to Inhibit the Endocytosis of Dl in Dorsal Cells

Endocytosis of Dl was studied in living stage 5 embryos by monitoring the internalization of anti-Dl antibodies injected in the extracellular perivitelline space. Anti-Dl antibodies (green) were coinjected with dextran (red) either ventrally (A–B′) or dorsally (C–D′) in wild-type (or control *hb-lacZ*-positive; β-galactosidase in blue) embryos (A, A′, and C–C′′) as well as *neur*^{IF65} (B and B′) and *Df(3L)Brd12* (D–D′′) mutant embryos. Mutant embryos were genotyped using *hb-lacZ* (blue) as a marker for the balancer chromosome. Fluorescent dextran was used to monitor injection into the perivitelline space and to label the plasma membrane.

(A–B′) Lateral views showing the internalization of anti-Dl antibodies by ventral cells in control embryos (A and A′; [A′] shows an enlarged view of the injected region) and in *neur*^{IF65} mutant embryos (B and B′). Endocytosis of Dl in ventral mesoderm is *neur* dependent.

(C–D′′) Dorsal views showing the localization of anti-Dl antibodies in control (C–C′′) and *Df(3L)Brd12* mutant embryos (D–D′′). Two different focal planes of the regions boxed in (C) and (D) are shown in (C′) and (D′) (surface views) and (C′′) and (D′′) (views taken at the level of the base of the nuclei). Dorsally injected anti-Dl antibodies remained at the surface of control embryos (compare [C′] to [C′′]), whereas they were efficiently endocytosed in *Df(3L)Brd12* embryos (D′′). Anterior is left.

early embryo and within proneural clusters during imaginal development (Boulianne et al., 1991). A second level of regulation is seen during asymmetric division of the SOP with the unequal partitioning of Neur at mitosis (Le Borgne and Schweisguth, 2003). In this study, we have identified a third level of regulation based on the inhibition of Neur by Brd family members. We find that all Brd family members (with the exception of m2) interact in the yeast two-hybrid assay with the E3 ubiquitin ligase Neur. The overexpression of *Brd* genes specifically inhibits Neur-dependent Notch signaling events and leads to a defect in Dl endocytosis. Conversely, loss of the *Brd-C* that contains six out of the ten *Brd* genes results in ectopic Dl endocytosis and ectopic expression of the Notch target gene *sim* in the early embryo. Finally, physical interaction of Tom with Neur appears to inhibit the interaction of Neur with its substrate Dl. We propose a model whereby proteins of the Brd family antagonize Neur-mediated Dl signaling by inhibiting the interaction of Dl with Neur.

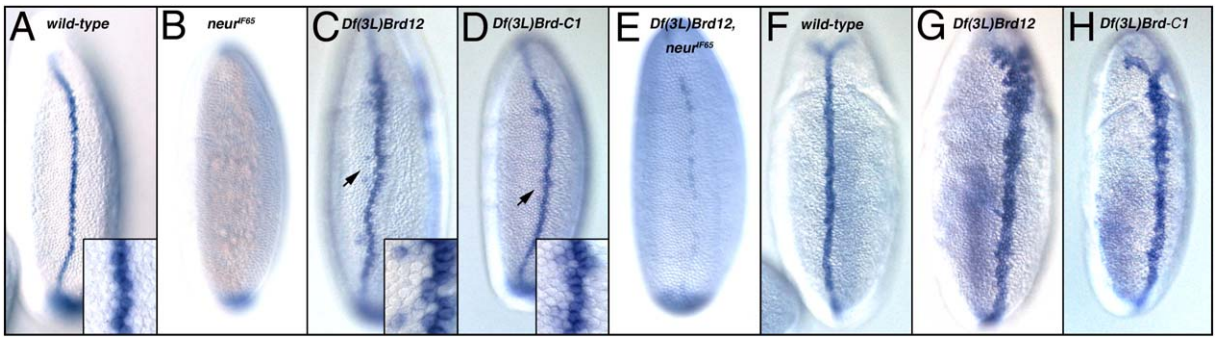

Figure 6. The *Brd-C* Is Required to Restrict the Expression of the *sim* Gene to a Single Cell Row

(A) Wild-type embryos express *sim* in a single row of cells during stage 5.

(B) Expression of the *sim* gene is not detected in *neur*^{IF65} mutant embryos at stage 5.

(C and D) Deletion of the *Brd-C* in *Df(3L)Brd12* (C) or *Df(3L)Brd-C1* embryos (D) leads to a weak ectopic expression of the *sim* gene dorsal to the mesectoderm (arrows point to the position of the insets in [C] and [D]).

(E) *Df(3L)Brd12, neur*^{IF65} double mutant embryos showed a partial rescue of the expression of the *sim* gene.

(F) Expression of the *sim* gene remained restricted to the two rows of mesectodermal cells that form the midline in stage 8 wild-type embryos.

(G and H) At this stage, the ectopic expression of the *sim* gene was clearly seen in *Df(3L)Brd12* (G) and *Df(3L)Brd-C1* (H) mutant embryos. Anterior is up.

A Model of Brd Function in DV Patterning

Precise positioning of the mesectoderm results from the integration of different activities that are more broadly distributed along the DV axis. The DV gradient of nuclear Dorsal is interpreted to establish large domains of gene expression (Rusch and Levine, 1996). The *twist* gene is expressed in a large ventral territory that encompasses the mesoderm, whereas the expression of the *snail* gene becomes restricted to the mesoderm. Twist and Dorsal activate the expression of the *sim* gene whereas Snail represses it. Neur-dependent Dl signaling in the mesoderm is thought to further restrict *sim* expression to cells in direct contact with the mesoderm (Morel and Schweisguth, 2000). The signaling activity of Dl is thought to be restricted to the mesoderm because its endocytosis is tightly restricted to the mesoderm in stage 5 embryos. While transcriptional regulation of *neur* in ventral cells likely contributes to this spatial regulation, it cannot on its own account for the mesoderm-specific regulation of Dl endocytosis. Indeed, high levels of transcripts are detected in ventral cells outside the mesoderm and low levels of transcripts are detected all around the embryo. This suggests that a posttranscriptional inhibitory mechanism exists to ensure that Neur is not active outside the mesoderm. We have shown here that Brd proteins inhibit Neur-mediated Dl endocytosis and Notch signaling in nonmesodermal cells. We have also shown that ectopic expression of Tom inhibits the endocytosis of Dl in the mesoderm. This suggests that the repression of the expression of *Brd-C* genes in the mesoderm is important for Neur to be active in this tissue. Inhibition of *Tom* expression (and possibly of the other *Brd-C* genes) in the mesoderm depends on the mesoderm-specific repressor Snail (Zaffran and Frasch, 2000). Accordingly, the ectopic expression of *Brd* genes in ventral cells of *snail* mutant embryos may explain the loss of Dl endocytosis and Notch activation that was previously observed in these embryos (Morel et al., 2003). We therefore suggest that the *Brd-C* genes represent the hypothesized Snail target gene X proposed to act as a negative regulator of Notch signaling and Dl endocytosis (Morel et al., 2003; reviewed in Stathopoulos and Levine, 2005). Thus, the sharp boundary of Snail expression appears to define the ventral limit of *Brd* family gene expression, hence the dorsal limit of Neur activity and Dl signaling. In summary, our data support a model whereby the *Brd* genes prevent ectopic Notch activation in the early embryo and contribute to DV patterning by restricting the mesectoderm territory to a single row of cells (Figure 7A).

A Possible Role for Brd Family Members in Lateral Inhibition

The function of the *Brd* genes is probably not restricted to the early embryo. Indeed, several *Brd* genes are also strongly expressed during early neurogenesis in the embryo as well as in the proneural clusters of the eye, leg, and wing imaginal discs (Knust et al., 1992; Lai et al., 2000a, 2000b; Leviten et al., 1997; Nagel et al., 2000; Singson et al., 1994; Wurmbach et al., 1999; Zaffran and Frasch, 2000). Proneural cluster expression of the *Brd* genes may be important to restrict, in space and/or time, the activity of Neur during the process of SOP determination. While Neur appears to be primarily expressed in the presumptive SOP, there is also evidence that Neur may also be expressed at low levels in non-SOP cells. In particular, low-level expression of Neur in non-SOP cells is occasionally seen using *neur^{P72}Gal4* (Bellaiche et al., 2004). We hypothesize that Brd may act to antagonize this low level of Neur activity in proneural cluster cells. Interestingly, the expression of the *neur* gene in SOPs is accompanied by the transcriptional repression of the *mα* gene by Su(H) in SOPs. The expression of other *Brd* family genes is excluded from SOPs, suggesting that they may also be repressed by Su(H) (Castro et al., 2005 and references therein). Conversely, the positive regulation of *Brd* gene expression by Notch in non-SOP cells correlates with a loss in Dl signaling activity in these cells. We therefore speculate that the *Brd* genes contribute to amplify an initially weak difference in Dl signaling activity between presumptive SOP and non-SOP cells (Figure 7B).

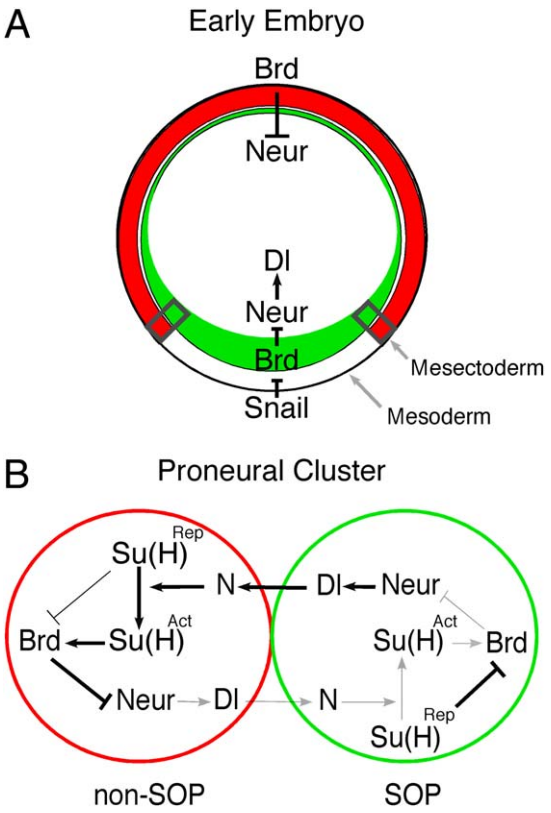

Figure 7. Possible Roles of the *Brd* Genes in Spatial Patterning

(A) Brd family members contribute to DV patterning. We propose that Snail-regulated expression of the *Brd* genes allows for mesoderm-specific Neur activity and Dl signaling, thereby promoting *sim* expression in the one-cell row contacting the mesoderm (gray box; see text for details).

(B) Hypothetical role of Brd family members in SOP selection. Lateral inhibition within proneural clusters is thought to involve a transcriptional feedback loop linking Notch activation in presumptive non-SOP cells and Dl signaling in the presumptive SOP. We propose that Neur and Brd may be part of such a feedback loop. Notch upregulates the expression of several *Brd* genes in non-SOP cells, whereas Su(H) represses *Brd* gene expression in SOPs. High levels of Brd in non-SOP cells would result in reduced levels of Neur-mediated Dl signaling, whereas low levels of Brd would allow for high levels of Neur-mediated Dl signaling.

The role proposed above for the *Brd* genes in lateral inhibition remains to be investigated. We found that the deletion of the *Brd-C* is largely embryonic lethal. However, a few homozygous *Brd-C1* escaper flies were observed. These *Brd-C1* flies showed no detectable defects in bristle density (data not shown). While this observation indicates that the *Brd-C* does not play an essential role in the process of SOP selection, the possibility remains that the *mα* and *m4* genes act redundantly with genes of the *Brd-C* in this process.

Inhibition of the Interaction of Dl with Neur by Brd Proteins

We have shown that all Brd family members (with the exception of m2) interact with Neur in the yeast two-hybrid assay. Interaction of Tom with Neur was further confirmed by coimmunoprecipitation experiments. Importantly, interaction of Tom with Neur correlated with a decrease in the amount of Dl immunoprecipitated by Neur, without affecting the levels of Neur and/or Dl. We note, however, that TomΔ2, which interacts weakly with Neur, can still inhibit interaction of Dl with Neur in this assay (data not shown). The ability of Tom to decrease the Dl-Neur binding in this assay led us to propose a model whereby Brd family members antagonize Neur-mediated Dl signaling by inhibiting the Neur-Dl interaction. This model is consistent with our observations that overexpression of Tom has no effect on Neur protein levels. It is also consistent with our observation that Tom blocks the activity of NeurC701S. The latter may act in a dominant-negative manner by titrating DSL ligands. Accordingly, Tom could prevent NeurC701S from titrating DSL ligands. Similarly, the failure of Tom to suppress the wing phenotype induced by Mib1C1205S is consistent with our observation that Tom does not bind Mib1 and cannot, therefore, prevent Mib1C1205S from titrating DSL ligands. Whether Brd family members inhibit Neur by competing with Dl for overlapping binding sites remains to be investigated.

Our studies have focused on the interaction between Neur and a single Brd family member for the sake of consistency. Tom was chosen because (1) it includes all four conserved motifs present in the various Brd family members; (2) it is the *Brd* gene that aligns best with the single *Anopheles Brd* gene; (3) its overexpression gives a strong gain-of-function phenotype; and (4) it is expressed at high levels in stage 5 embryos. Whether all Brd family members similarly act by inhibiting the Neur-Dl interaction remains to be fully investigated. Because all Brd family members (with the exception of m2) have been shown to inhibit Neur-mediated Notch signaling, it is likely that all Brd family members similarly inhibit Neur. This in turn raises the question of the role of the two additional conserved motifs found at the C terminus of Ocho, Tom, mα, m4, and m6 that are also conserved in the single *Bombyx* and *Anopheles Brd* homologs.

While Neur has homologs in vertebrates and *Xenopus* Neur has been suggested to regulate Dl signaling during early neurogenesis (Deblandre et al., 2001), no obvious homologs of the *Brd* genes are detectable in vertebrate sequenced genomes. This does not, however, exclude the possibility that vertebrate genes encoding Brd-like inhibitors exist. Indeed, motif 2 of Brd may be too short to reliably detect possible *Brd* homologs in vertebrate genomes by sequence alignments.

In conclusion, we have shown that Brd family members interact with Neur and block Neur-mediated Dl endocytosis. The activity of the *Brd-C* is required to spatially restrict Dl signaling along the DV axis in the early embryo.

Experimental Procedures

Drosophila Stocks

The *Df(3L)Brd12* and *Df(3L)Brd15* deficiencies were obtained from the Bloomington Stock Center. The *Df(3L)ED217* was obtained from the Szeged Stock Center. The P insertion lines WH f02655 and XP d02180 used to generate the *Df(3L)Brd-C1* deficiency were produced by Exelixis (San Francisco, CA) and obtained from S. Artavanis-Tsakonas. Flp-mediated recombination was carried out as described in Thibault et al. (2004) (Figure S3). The following transgenic lines were used in this study: UAS-Tom (Lai et al., 2000a; Nagel et al., 2000), UAS-m2 (unpublished; gift of A. Preiss), UAS-mα (Apidianakis et al., 1999), and UAS-m4::GFP (Apidianakis et al., 1999).

UAS-Mib1C1205S and UAS-NeurC701S were generated in this study. The C-to-S mutations were introduced into the corresponding cDNA by PCR and mutant cDNAs were cloned into the pUAST vector (cloning details available upon request). Expression of Mib1C1205S and NeurC701S did not rescue the *mib1* and *neur* mutant phenotypes, respectively (S. Hamel and F.S., unpublished data). The following Gal4 drivers were used: *ap*-Gal4, neur^{P72}Gal4, *Ser*-Gal4, *dpp*-Gal4, *ptc*-Gal4 (described by and available from the Bloomington Stock Center), and *matαTub-Gal4VP16 67C;15* (obtained from D. St Johnston via T. Lecuit). Clones of *neur^{IF65}* mutant cells were generated in pupae of the following genotype: *ap*-Gal4/UAS-FLP; FRT82B ubi-nlsGFP/FRT82B *neur^{IF65}*. Genotyping of stage 5 embryos relied on the early expression of the *hb-lacZ* transgene carried by the TM3 *hb-lacZ* balancer (obtained from E. Wieschaus).

Immunostainings

Notum dissection and antibody staining were performed as previously described in Gho et al. (1996). Immunostaining of embryos and dissected imaginal discs was done using standard procedures. For the analysis of cross-sectioned embryos, immunostained embryos in mounting medium were genotyped under the microscope and placed individually onto a 5 μl drop of mounting medium. Embryos were then individually sliced using a sharp scalpel blade. Embryo slices were mounted flat on a slide and imaged using confocal microscopy. The following antibodies were used: mouse anti-Dl (C594-9B; 1:1000; Developmental Studies Hybridoma Bank [DSHB], under the auspices of the NICHD, University of Iowa, Iowa City), mouse anti-Notch ECD (C548.2H; 1:1000; DSHB), guinea pig anti-Dl (GP581; 1:3000; M. Muskavitch), rabbit anti-Twist (1:3000; S. Rorth), guinea pig anti-Sens (1:3000; H. Bellen), rabbit anti-β-galactosidase (1:1000; Cappel, Durham, NC), rabbit anti-Prospero (1:2000; Y.N. Jan), mouse anti-Cut (1:500; DSHB), rabbit anti-Spdo (1:2000; J. Skeath), and rat anti-Elav (1:5; DSHB).

Antibody Uptake Assay

The anti-Dl uptake assay was carried out in nota dissected from 16.5 hr after puparium formation pupae as described (Le Borgne and Schweisguth, 2003). For the anti-Dl uptake assay in embryos, stage 5 embryos were dechorionated using bleach, extensively rinsed in water, and lined up onto double-sided tape with the ventral (or dorsal) side facing the injection needle. Embryos were injected under Voltalef 10S oil (Prolabo, Fontenay-sur-Bois, France). The injection mix consisted of a 1:4 mix of tetramethylrhodamine dextran 3000 (20 mg/ml; Molecular Probes, Invitrogen, Cergy Pontoise, France) and mouse anti-Dl (C594.9B1; DSHB). Injection into the perivitelline space was directly monitored using fluorescent dextran dye. Injected embryos were incubated 10–15 min at 18°C, and were then hand-brushed into a heptane/4% formaldehyde fixative mix. Embryos were fixed for 30 min, rinsed in PBS, stuck onto double-sided tape, and hand-devitellinized using a tungsten needle. Embryos were then postfixed 15 min in 4% formaldehyde in PBS once prior to incubation with primary and secondary antibodies.

Yeast Two-Hybrid Screen

A yeast two-hybrid screen was conducted as previously described (Kolonin et al., 2000). A fragment of Neur encoding the two NHRs (amino acids 93–532) was cloned into the pMW103 plasmid (gift of E. Golemis) to create a fusion protein with the LexA DNA binding domain and transformed into the RFY206 yeast strain (*Mat a*). The RFLY1 embryonic cDNA library (fused to B42 transcription activation domain; gift of R. Finley) was amplified and transformed into the RFY231 yeast strain (*Mat* α). We screened 7×10^6 potential interactors (5-fold library coverage) and assayed ability to activate *LEU2* and *LacZ* reporters.

Coimmunoprecipitation

Expression vectors containing Dl (Polyoma-tagged), NeurΔRF, NeurΔNHR1, and Mib1ΔRF (all MYC-tagged) were obtained from E. Lai (Lai et al., 2001). One million HEK293 cells were transfected with 1 μg of plasmids. Total DNA concentration was kept constant by using a control plasmid encoding a Flag-tagged β-galactosidase. Forty-eight hours after transfection, cells were lysed in 330 μl of 0.5% Triton buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol, 0.5% Triton X100), 0.5 mM DTT, 1× protease inhibitors cocktail EDTA-free (Roche, Meylan, France). Five microliters of mouse anti-MYC 9E10 (Roche) were added to the extract and incubated 1 hr. This was followed by the addition of 25 μl of washed protein G beads (Roche) for 2 hr at 4ºC, rotating. For Tom-Neur immunoprecipitation, a polyclonal rabbit anti-MYC (Upstate Biotechnology, Euromedex, Mundolsheim, France; 06-549; 3 μl) was used followed by protein A beads. Beads were washed seven times with 0.5% Triton buffer or with RIPA buffer for Dl coimmunoprecipitation (1% NP40, 0.1% SDS, 150 mM NaCl, 1% sodium deoxycholate, 1 mM EDTA, 50 mM Tris [pH 7.4]), 0.5 mM DTT, 1× protease inhibitors cocktail EDTA-free. Immunoprecipitates were analyzed on either Tris-glycine PAGE or Tris-tricine PAGE (to detect Tom) gels. Western blots were performed using goat anti-Dl (1:200; Santa Cruz Biotechnology, Santa Cruz, CA; dc-19), mouse anti-Flag (1:1000; Sigma, Lyon, France; F9291), or mouse anti-MYC 9E10 (1:1000; Roche; 11 667 149 001). Coimmunoprecipitations were repeated five times, each with similar results.

Supplemental Data

Supplemental Data include five figures and are available at http://www.developmentalcell.com/cgi/content/full/10/2/245/DC1/.

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